Developmental evidence helps resolve the evolutionary origins of anther appendages in *Globba* (Zingiberaceae)

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Abstract:

Globba is one of the largest genera in the primarily tropical Zingiberaceae. The number of anther appendages is highly diagnostic and has been used along with molecular characters to define subgenera and sections. Four main types of anther morphology are recognized: without appendages and with two, four and six appendages. The six-appendaged anthers are reported here for the first time. Appendages arise from two dorsal ledges that flank the broad connective. Development of two-appendaged and four-appendaged species differs from inception. Previous suggestions that either the proximal or distal appendages of four-appendaged anthers have been lost in two-appendaged species are thus not supported. Early development of six-appendaged anthers is similar to that of four-appendaged species, but two additional, small appendages develop on the ledges between the first-formed appendages. This yields three appendages on each side (six overall). The four appendages of *G. geoffrayi* differ from all other species in having distal appendages that are much smaller and develop later than the proximal appendages. Development thus suggests that the state in *G. geoffrayi* evolved from a two-appendaged ancestor. Incorporating this information into a phylogenetic character plot of the number of appendages shows that the possession of two appendages is the most likely plesiomorphic state of the genus, although support for this hypothesis is weak. Our study clarifies the origin and complexity in the development of anther appendages in *Globba* and highlights their significance in infrageneric relationships in *Globba*. Two appendages have probably likely arisen at the base of *Globba*, linked with the presence of a prominent ledge, with variable extensions and reductions of the number of appendages in the various subgenera and sections.

Keywords: connective | floral development | Globbeae | phylogeny

Article:

INTRODUCTION

Globba L. is one of the largest genera in the primarily tropical Zingiberaceae and comprises *c*. 100 species distributed throughout tropical (and parts of subtropical) South-East Asia (Leong-Škorničková & Newman, 2015). The distribution of the genus ranges from India to southern China, south and east to the Philippines and New Guinea (Schumann, 1904). *Globba* [including *Mantisia* (Sims) K.J. Williams], *Gagnepainia* K.Schum. (three species) and *Hemiorchis* Kurz (three species) comprise Globbeae, one of the four traditionally recognized tribes of Zingiberaceae (e.g. Schumann, 1904; Larsen *et al.*, 1998; Williams, Kress & Manos, 2004). Kress, Prince & Williams (2002) linked Globbeae with Zingibereae in subfamily Zingiberoideae based on molecular data. The molecular analysis of Williams *et al.* (2004) showed that the traditionally recognized genus *Mantisia* is embedded in *Globba*, sister to a clade of Indo-Burmese species, a conclusion that is further supported by a number of vegetative and floral characters.

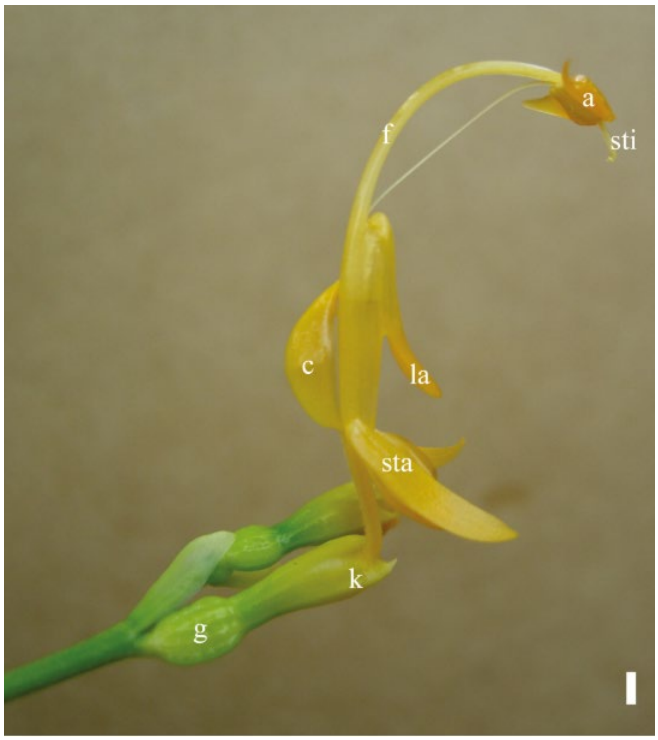

Figure 1. Lateral view of mature flower of *Globba pendula*, an example with two appendages. Abbreviations: a, anther; c, corolla lobe; f, filament; g, ovary; k, calyx lobe; la, labellum; sta, lateral staminode; sti, stigma. Scale bar = 1 mm.

The most important character shared by all Globbeae is a unilocular ovary with parietal placentation, as opposed to a trilocular ovary with axile placentation in most of the family (Schumann, 1904; Larsen *et al.*, 1998). However, this distinction is not absolute as some genera of Zingiberaceae have an intermediate placentation type (e.g. *Scaphochlamys* Baker: Kirchoff 1998). Zingiberaceae are among the most diverse families of monocots in terms of floral morphology and pollination systems (Endress, 1994; Specht *et al.*, 2012). Flowers of all Zingiberaceae are characterized by a single prominent fertile stamen with a broad flattened anther that encloses the style. *Globba* and *Gagnepainia* are distinctive in Zingiberaceae in having

a large arching filament linked to a small labellum (Fig. 1; Williams *et al.*, 2004; Leong-Škorničková & Newman, 2015). In *Globba* the filament is greatly elongated and is as long as, or longer than the floral tube and staminodes. The anthers of most *Globba*spp. are unique in that they bear two to four, well-developed appendages along the sides of the anthers (Williams *et al.*, 2004). The reported number of anther appendages (zero, two or four) is highly diagnostic and has been used extensively in the infrageneric classification of *Globba* (Horaninow, 1862; Schumann, 1904; Larsen, 1972; Williams *et al.*, 2004).

Table 1. Source of investigated species with reference to the figures

Subgenus	**Section***	**Species**	**RBGE Living collection accession numbers**	**Plant origin and collection voucher****	**Number of appendages**	**Figures**
Mantisia	*Haplanthera*	*Globba racemose* Sm.	19920039	Nepal	0	Figs 2A, 3A–H
Mantisia	*Haplanthera*	*G. racemosa*Sm.	19991625	Thailand	0	Figs 2B, 3I–L
Mantisia	*Haplanthera*	*G. sessiliflora*Sims	-----	Williams *et al.* (2004)	0	their Fig. 3
Mantisia	*Mantisia*	*G. spathulate* Roxb.	-----	Box & Rudall (2006)	2	their Fig. 6E
Ceratanthera	*Ceratanthera*	*G. pendula* Roxb. (synonym *G. fasciata* Ridl.)	19860743	Thailand, Nakhon Si Thammarat - *Newman & Škorničková 1992*	2	Fig. 2E, 4G–L
Ceratanthera	*Ceratanthera*	*G. pendula* Roxb.	20081102	Vietnam, Dong Nai Prov. - *Newman & Poulsen 2435*	2	Fig. 1, 2F
Ceratanthera	*Ceratanthera*	*G. geoffrayi* Gagnep.	20100754	Cambodia	4	Fig. 2H, 5A–F
Globba	*Nudae*	*G. paniculate* Valeton	20010323	Indonesia, Aceh - *Newman & Škorničková 2047*	2	Fig. 2C, 4A–F
Globba	*Nudae*	*G. campsophylla* K.Schum.	19972553	Philippines, Luzon - *Newman & Škorničková 2006*	2	Fig. 2D
Globba	*Nudae*	*G. albiflora* Ridl. var. *aurea* Holttum	20060805	Laos, Bolikhamxai - *Newman et al. LAO 1529*	2	Fig. 2G
Globba	*Nudae*	*G. flagellaris* K. Larsen	19991624	Thailand - *Newman & Škorničková 2048*	4	Fig. 2K, L
Globba	*Globba*	*G. candida*Gagnep.	20091380	Laos	4	Fig. 2M, N
Globba	*Globba*	*G. siamensis* (Hemsl.) Hemsl.	20060813	Laos, Bolikhamxai - *Newman et al. LAO1533*	6	Fig. 2Q, 6A–H
Globba	*Sempervirens*	*G. patens* Miq.	19680620	Malaysia, Selangor/ Pahang border - *Newman & Škorničková 2017*	4	Fig. 2P
Globba	*Sempervirens*	*G. propinqua* Ridl.	19841210	Malaysia, Sabah.	6	Fig. 2R, S
Globba	*Sempervirens*	*G. atrosanguinea* Teijsm. & Binn.	19820784	Malaysia, Sarawak - *Sinclair & Newman & Škorničková 2019*	6	Fig. 2T, 5I–P
Globba	*Sempervirens*	*G. cernua* Baker	-----	Box & Rudall (2006)	4	their Figs 1, 2I
-----	------	*Hemiorchis burmanica* Kurz	19991652	Chittagong, Bangladesh - *Newman 1001*	0	Fig. 7E, F
-----	-----	*Gagnepainia godefroyi* (Baill.) K.Schum.	20010444	Thailand - *Tan 5, 15, 16, Newman*	0	Fig. 7C, D
-----	-----	*Gagnepainia harmandii* K.Schum.	19991163	Thailand, Newman 931	0	Fig. 7A, B
-----	-----	*Cautleya spicata* (Sm.) Baker	-----	Ngamriabsakul (2005)	0	his Figs 3, 7, 9, 11

*According to Williams *et al.* (2004); **refers to herbarium voucher at RBGE (E) when available

Floral developmental investigations are known to provide useful characters for phylogenetic studies (e.g. Kirchoff, 1988, 1998; Ronse De Craene & Smets, 1999; Ronse De Craene *et al.*, 1998; Bachelier, Endress & Ronse De Craene, 2011; Prenner & Cardoso, 2017). The primary goal of this study is to document the structure and development of the appendages of *Globba*, and explore their origins and evolution. The findings of this investigation, combined with results of previous phylogenetic studies, will provide insight into the evolution of Globbeae and shed light on the evolution of developmental patterns. This study sheds particular light on (1) the homology of the appendages in two- and four-appendaged species and (2) the evolution of the various numbers of appendages in the genus. We also provide the first report of six-appendaged *Globba* spp.

MATERIAL AND METHODS

Fresh flowers of 15 *Globba* spp., two *Gagnepainia* spp. [*G. godefroyi* (Baill.) K.Schum. and *G. harmandii* K.Schum.] and *Hemiorchis burmanica* Kurz were collected by LRDC from the living collections in the Royal Botanic Garden Edinburgh, fixed and stored in 70% ethanol (Table 1).

For examination of anther appendages using light microscopy (LM), we dissected mature flower buds in 70% ethanol under a Zeiss Stemi SV6 dissecting microscope. At least five flowers were investigated per taxon. Photomicrographs were taken using a Zeiss Axiophot photomicroscope fitted with a Zeiss Axio-Cam digital camera.

For examination of the development of anther appendages using scanning electron microscopy (SEM), we dissected floral buds in 70% ethanol under a Zeiss Stemi SV6 dissecting microscope. After being washed repeatedly in 70% ethanol, floral buds of different stages were dehydrated in an ethanol-acetone series. Material was then critical-point-dried with CO_2 using a Quorum K850 critical point drier. Materials mounted on SEM stubs were coated with platinum using an Emitech K575x sputter coater and observed under a Leo Supra 55VP SEM. We investigated the following taxa for the earlier stages of appendage differentiation and compared similar stages of development: *Globba racemosa* Sm., *G. paniculata* Valeton, *G. pendula* Roxb., *G. geoffrayi* Gagnep., *G. marantina* L., *G. adhaerens* Gagnep., *G. siamensis* (Hemsl.) Hemsl., *G. atrosanguinea* Teijsm. & Binn., *Hemiorchis burmanica*, *Gagnepainia harmandii* and *Gagnepainia godefroyi* (Table 1).

Ancestral state reconstruction analyses were performed in Mesquite version 3.51 (Maddison & Maddison, 2018) using parsimony and maximum likelihood approaches. Tree topology was obtained by manually constructing a phylogenetic tree of the studied species based on the combined ITS/*matK* analysis of Williams *et al.*(2004: figs 10, 11). Additional species studied here but not considered by Williams *et al.* (2004) were manually placed on the tree using the topology of the Bayesian consensus tree from the MSc thesis of M. Pospíšilová (2012) (*G. campsophylla* K.Schum., *G. flagellaris* K.Larsen, *G. candida* Gagnep., *G. siamensis*). Support for the position of these additional species was strong and did not affect the original tree topology. We included character state data on *G. sessiliflora* Sims, which lacks appendages, in the analysis based on figure 3 in Williams *et al.* (2004) (Table 1). We also included data on the two species observed by Box & Rudall (2006) [*G. cernua*Baker (four appendages) and *G. spathulata* Roxb. (two appendages)], based on their figures (Table 1). We chose three species as

outgroups from the immediate sister groups of *Globba* as determined by both Kress *et al.* (2002) and Williams *et al.* (2004): *Hemiorchis burmanica*, *Gagnepainia godefroyi* and *G. harmandii.* We chose *Cautleya spicata* (Sm.) Baker as a more distant outgroup based on the placement of this taxon by Kress *et al.* (2002) and the developmental study of Ngamriabsakul (2005). Images in Ngamriabsakul (2005) show that this species lacks *Globba*-like appendages.

We investigated the evolution of the following characters: (1) presence or absence of anther appendages; (2) number of appendages and (3) the number of appendages formed early in development. Information on character state coding is given in the Results.

RESULTS

Morphology of the mature anther appendages

In *Globba* there is a single fertile stamen, which is the median adaxial member of the inner whorl. At anthesis, the large fertile stamen dominates the floral architecture. The filament is broadly flattened with multiple veins and is inflexed, forming a curved supportive structure stretching the style like the string of a bow (Fig. 1). The anther is large, dithecal and tetrasporangiate. Most *Globba* spp. have pronounced and characteristic lateral appendages, varying in number and position according to species (Table 1; Figs 1, 2). The broad filament possesses several vascular traces, but no vasculature was observed in the appendages (Fig. 2).

No lateral anther appendages were found in *G. racemosa* (Fig. 2A, B). Four taxa (*G. paniculata*, *G. campsophylla*, *G. pendula* and *G. albiflora* var. *aurea*) possess only two appendages (Fig. 2C–G). The first three of these taxa possess two broad appendages, whereas the appendage is relatively slender in the last. Unlike all other species investigated, the stigma of *G. pendula* is slender and extends far beyond the anther (Fig. 2E, F).

We observed anthers with four triangular lateral appendages in seven species (*G. geoffrayi*, *G. adhaerens*, *G. winitii*, *G. flagellaris*, *G. candida*, *G. marantina* and *G. patens*; Fig. 2H–N). In most of these species, the distal two appendages and proximal two appendages are not obviously different in size. In *G. geoffrayi*, however, the proximal anther appendages are small and slender, and the distal appendages are much smaller than the proximal ones (Fig. 2H). For this reason, we initially coded *G. geoffrayi* as having its own type of appendages (the *G. geoffrayi* type).

In addition to the appendages, several species with four appendages also possess irregular dentations (lobes) in their distal or proximal appendages. These dentations are especially evident in *G. patens* and *G. marantina* (Fig. 2O, P), but are also present in *G. adhaerens* (Fig. 2I). Based on the late development of these dentations (see below), we interpret these species as possessing four appendages. In four-appendaged species, the two appendages of each pair can also be fused at the base. This condition is especially evident in *G. candida* (Fig. 2K, L) and *G. marantina* (Fig. 2O, P).

Three species, *G. siamensis*, *G. propinqua* Ridl. and *G. atrosanguinea*, were found to possess six appendages, including a smaller appendage between the two larger appendages on each side (Fig. 2Q–T). However, in *G. atrosanguinea* the smaller appendage is occasionally absent.

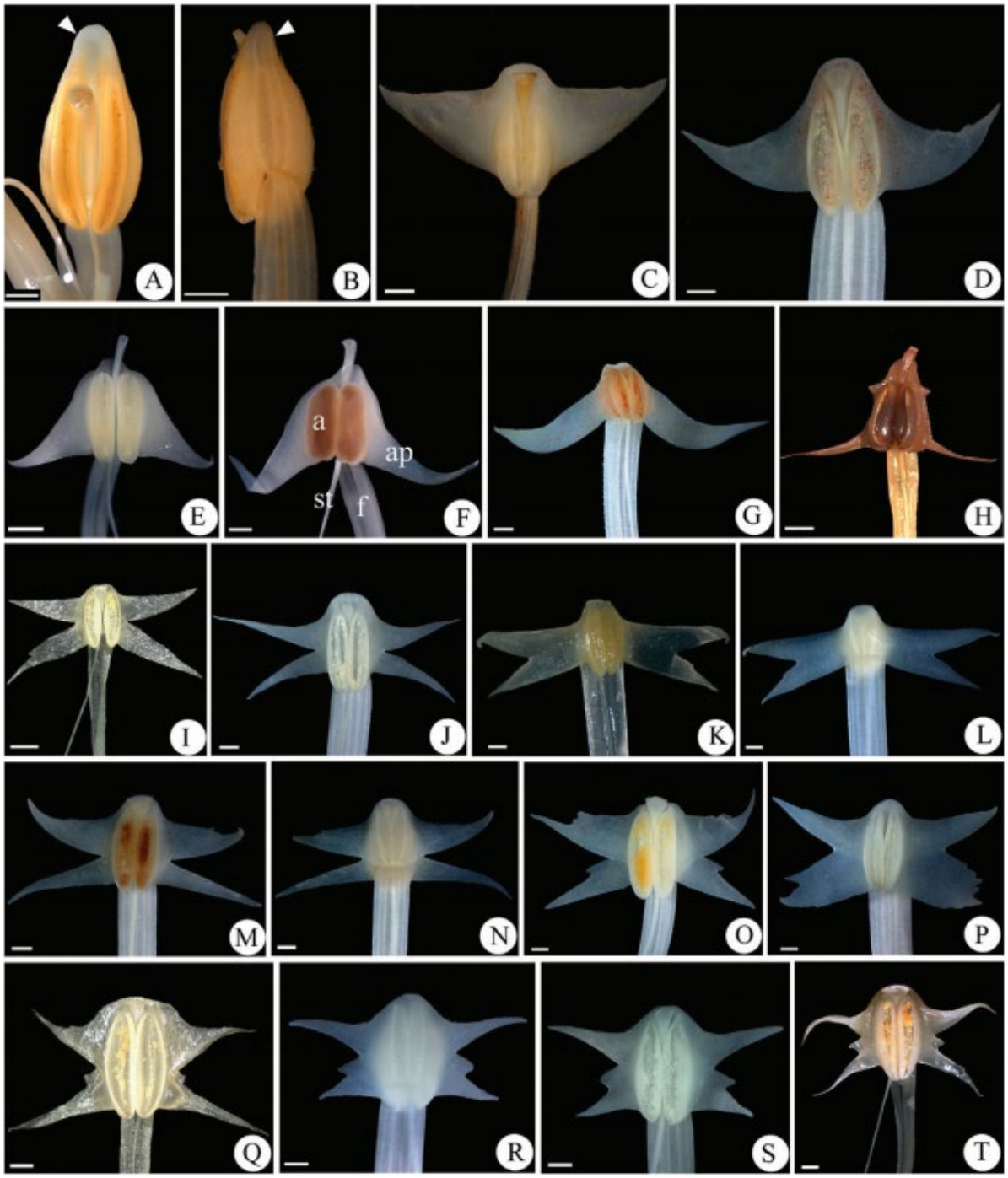

Figure 2. The morphology of *Globba* anther appendages at anthesis under a light microscope; all views from adaxial side except B, L, N, R from abaxial side. *2A–B*, Anthers without appendages: *G. racemosa*; C–G, Anthers with two appendages. C, G. *paniculata*; D, *G. campsophylla*; E–F, *G. pendula*; G, *G. albiflora*var. *aurea*; H–P, Anthers with four appendages. H, *G. geoffrayi*; I, *G. adhaerens*; J, *G. winitii*; K, L, *G. flagellaris*; M, N, *G. candida*; O, *G. marantina*; P, *G. patens*; Q–T, Anthers with six appendages. Q, *G. siamensis*; R, S, *G. propinqua*; T, *G. atrosanguinea*. *Globba atrosanguinea* has a variable state, with four to six appendages; the second possibility is shown here. Abbreviations: a, anther; ap, appendage; f, filament; st, style. Scale bar = 1000 μm (Fig. 2A–C, E, H) and 500 μm (Fig. 2D, F–G, I–T).

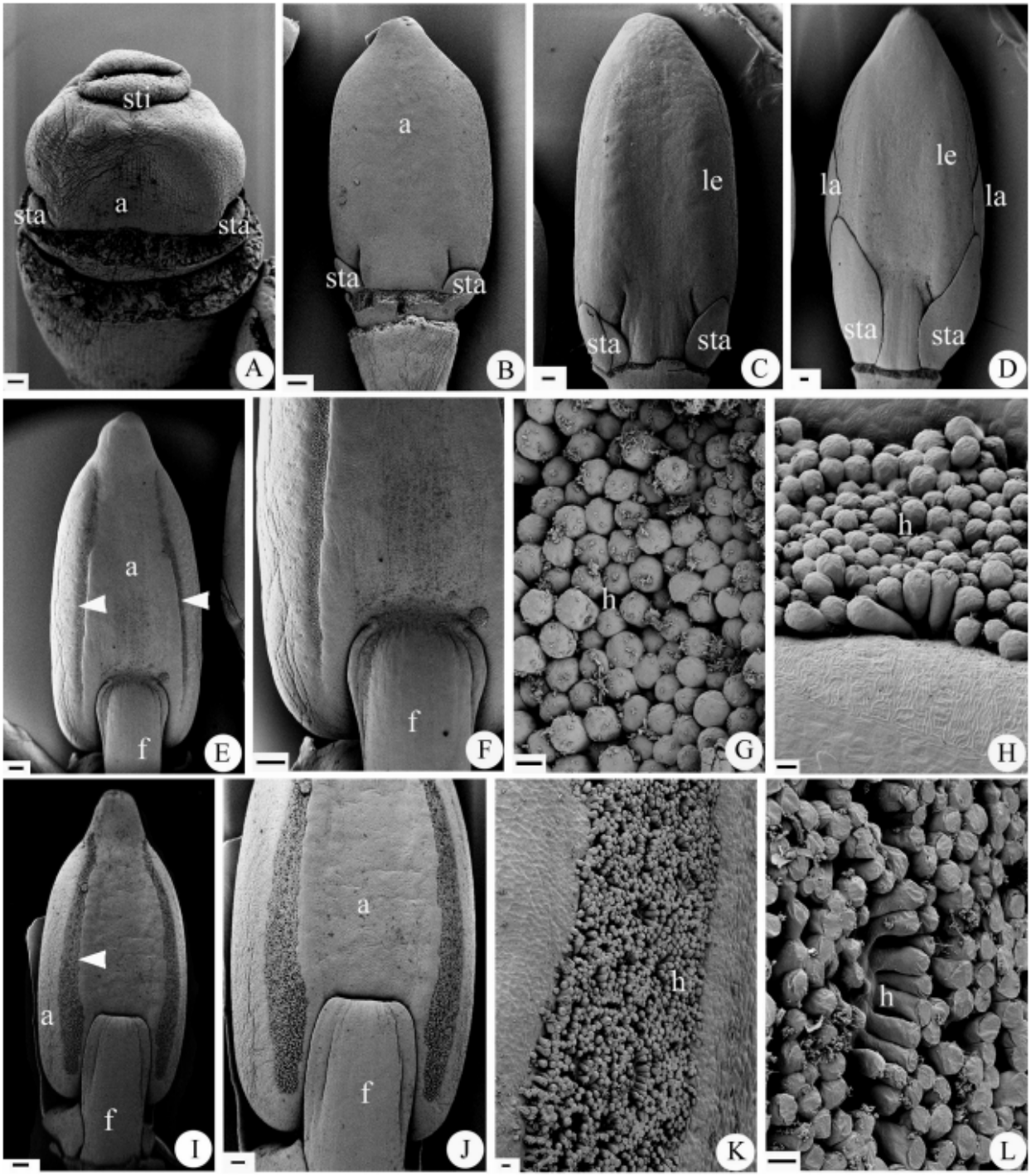

Figure 3. Scanning electron micrographs of the development of *Globba racemosa* without anther appendages. All views from adaxial side. A–B, Development of smooth anthers with expanding connective tip. C–D, Initiation of weak parallel ledges. E, F, Development of narrow stripes with glandular trichomes. G–H, Apical and lateral view of glandular trichomes. I, Nearly mature anther with abaxial stripes. J–L. Progressive magnification of stripes with glandular trichomes. Abbreviations: a, anther; f, filament; h, glandular hair; la, labellum; le: ledge; sta, lateral staminode; sti: stigma. Scale bar = 200 μm (E, F, I), 100 μm (B–D, J), 10 μm (G, H, K, L), 30 μm (A).

Development of anther appendages

Globba *species without anther appendages*

The anthers of *G. racemosa* are broad rectangular structures with a prominent connective extension (Fig. 2A, B). No clear anther appendages appear during stamen development of this species (Fig. 3A–D). Along the margins of the connective, in the area that separates the pollen sacs from the connective at the back of the anther, two narrow ledges develop parallel to the margin of the anther (Fig. 3C, D). Each ledge is flanked towards the connective by a prominent stripe covered with cylindrical trichomes (Fig. 3E–L, arrowhead). In the later stages of development, the ledges stop growing, whereas the rest of the anther continues to enlarge, leaving only the two stripes with trichomes visible. The trichomes are in the shape of inverted cones (Fig. 3G, H, K, L).

Globba *species with two anther appendages*

In species with two appendages, the appendages arise from two abaxial ledges situated on the margins of a broad connective. The young stamen is rather smooth at first (Fig. 4A, G). As development proceeds, each of the two ledges expands outward to form a triangular appendage on the proximal part of the anther close to the insertion of the filament (Fig. 4B, C, I, J). The appendages expand further and enfold the filament tightly as two wings (Fig. 4D, E, J, K). The filament elongates faster than the surrounding perianth, causing it to curve distally. Development of the wings coincides with the bending of the filament, which is pushed upwards between the appendages (Fig. 4E, J, K). At anthesis the appendages elongate and become flat triangular wings extending along the whole anther margin (Fig. 4F, L).

Globba *species with four anther appendages*

Just as in species with two appendages, the young anther of this type is smooth (Fig. 5A). Two developmental patterns can be detected. In *G. geoffrayi*, the development of the appendages starts with the development of narrow ledges (Fig. 5B). Two small emergences develop in the proximal part of the ledge similar to species with two appendages (Fig. 5C); two additional appendages emerge later in the distal part of the anther (Fig. 5C, D). The proximal appendages grow rapidly into slender extensions that enfold the curving filament. The distal appendages grow more slowly and are much smaller than the proximal ones at maturity (Fig. 5C–F).

In other species with four ledges (*G. adhaerens*, *G. marantina*), two narrow ledges develop first and four flattened appendages emerge almost simultaneously on these ledges in the medio-proximal part of the anther, at the time the filament start curving (Fig. 5G, H, M, N). The appendages grow equally and embrace the filament tightly, and the filament is pushed upwards and bends in its upper part (Fig. 5H–K, N–P). The appendages elongate and become flattened wings at maturity (Fig. 5L, P). The late development of secondary appendages (lobes) leads to the wings becoming irregular at their margins (Figs 2O, P, 5L). The two wings on each side of the anther are unequal and may be separated by deep divisions (Fig. 2I, J, L, M, N), or they may be connected by their margins (Fig. 2K, O, P).

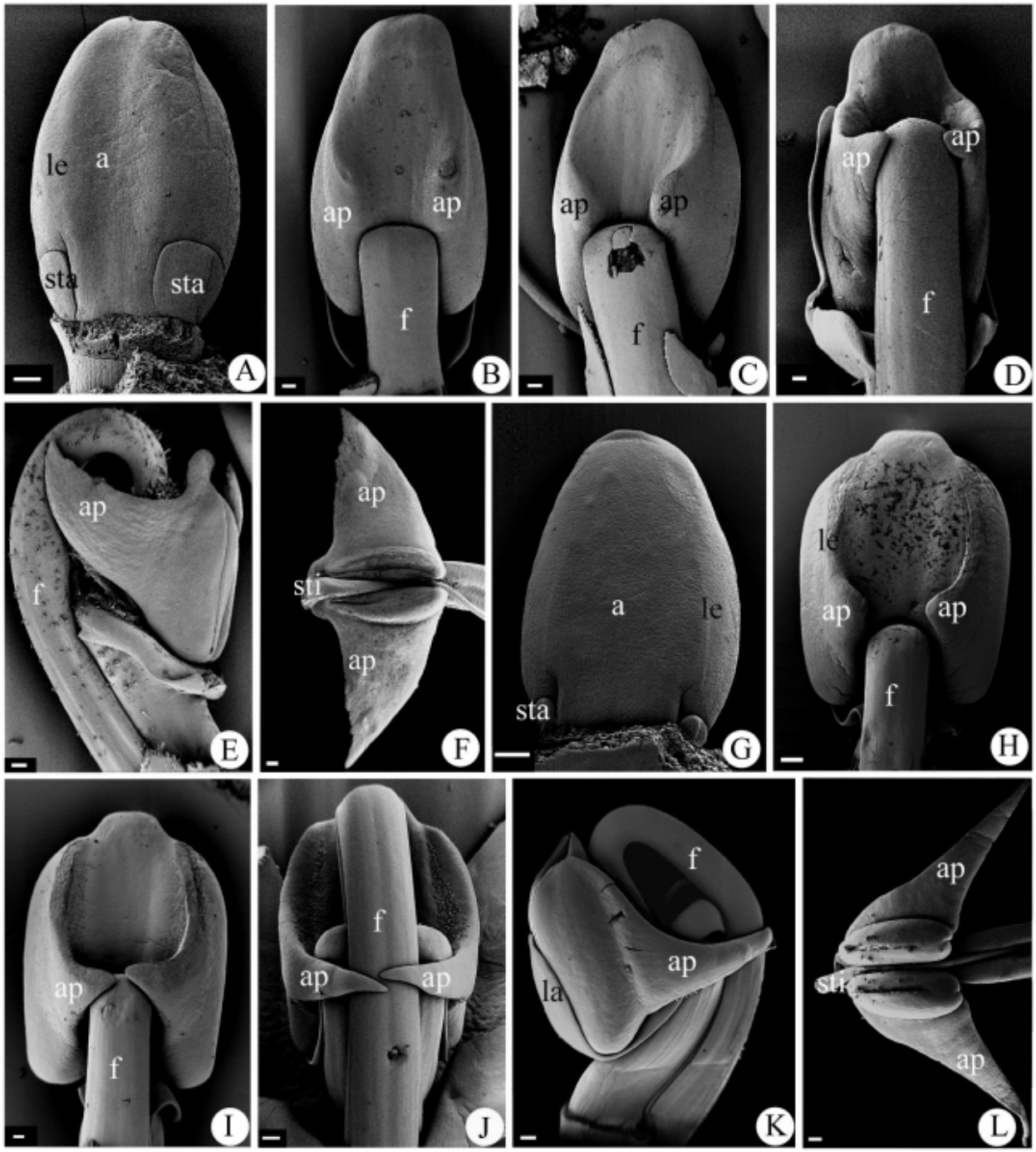

Figure 4. Scanning electron micrographs of the development of *Globba* species with two anther appendages. A–D, G–J: views from adaxial side; F, L: views from abaxial side. A–F, *G. paniculata*; G–L, *G. pendula*. A, G, Smooth young stamens. B–C, H, Two prominent appendages develop close to the filament. D–E, I–K, The appendages expand and enfold the anther attachment. F, L, The appendages elongate and become flat triangular wings. Abbreviations: a, anther; ap, appendage; f, filament; la, labellum; le, ledge; sta, lateral staminode; sti, stigma. Scale bar = 100 μm (A–D, G, I), 200 μm (E, F, H, J–L).

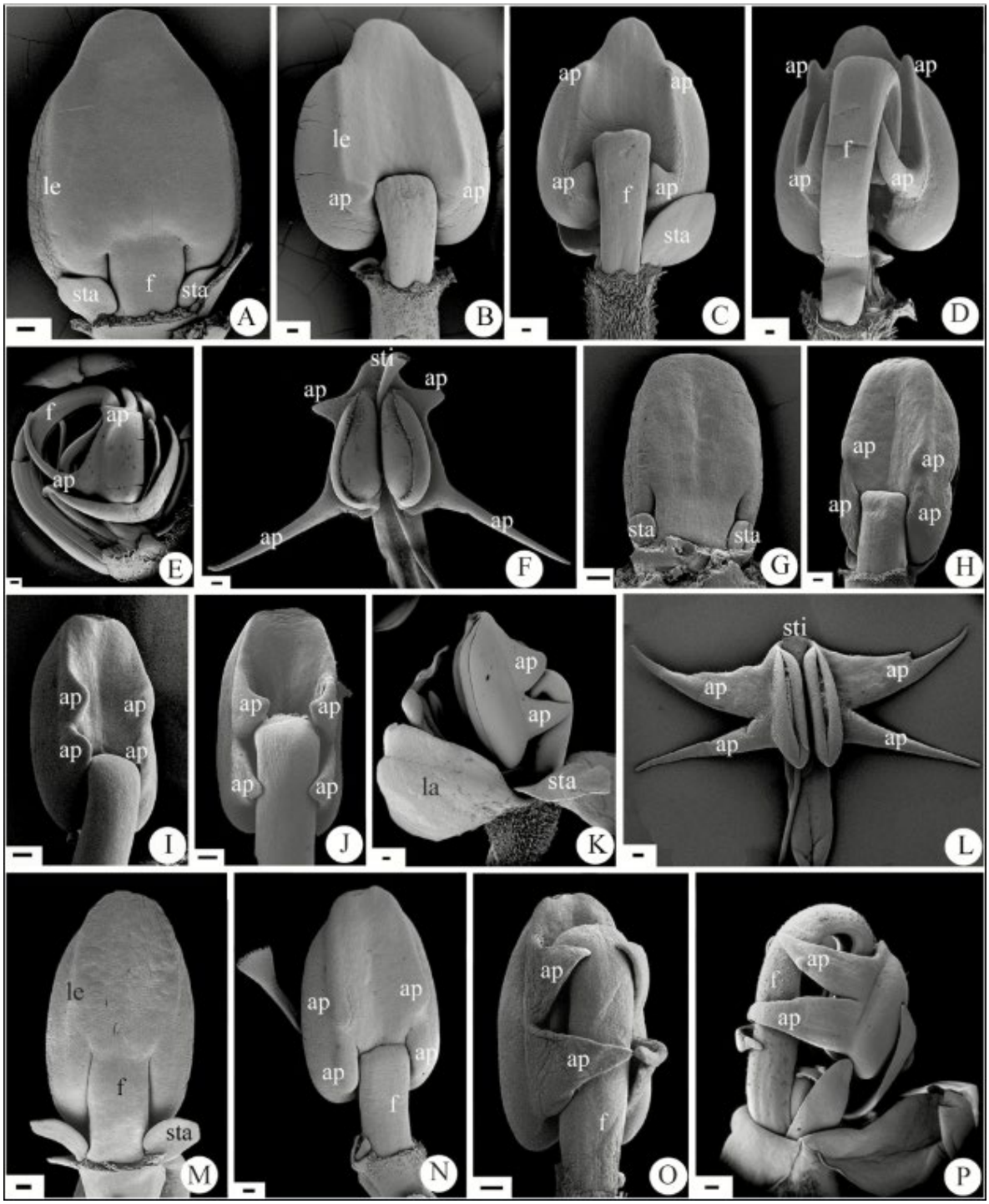

Figure 5. Scanning electron micrographs of the development of *Globba* species with four anther appendages. A–D, G–J, M–N: views from adaxial side; F, L: views from abaxial side. A–F, *G. geoffrayi*; G–L, *G. marantina*; M–P, *G. adhaerens*. A, G, M. Smooth young anthers. H, N, Four triangular appendages emerge in the proximal-middle part of the anthers. H–K, N–P, The appendages grow equally and hug the filaments tightly. L, F, The appendages elongate and become flattened wings. Abbreviations: ap: appendage; f: filament; la, labellum; le, ledge; sti: stigma; sta: lateral staminode. Scale bar = 100μm (A–D, G, H, K, M, N), 200 μm (E, F, I, J, L, O, P).

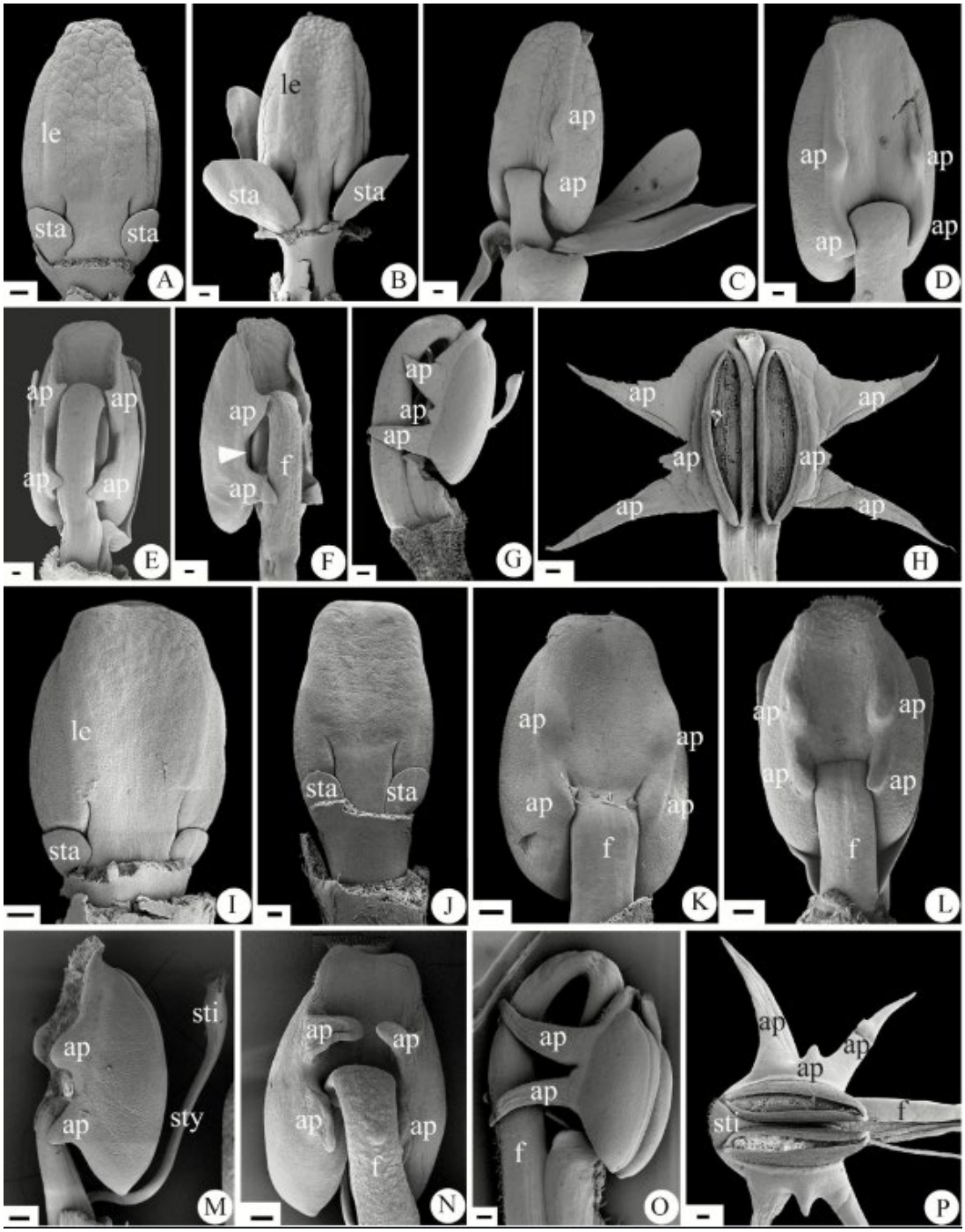

Figure 6. Scanning electron micrographs of the development of *Globba* species with six anther appendages. A–E, I–L: views from adaxial side; H, P: views from abaxial side. A–H, *G. siamensis*; I–P, *G. atrosanguinea*. A–B, I–J, Young anthers with irregular abaxial surface. C–D, K–L, Four triangular appendages emerge simultaneously. E–F, M–N, The appendages develop to four prominent flaps. G, O, The appendages start to hug the filaments and two smaller appendages emerge in between. H, P, Adaxial view of anther with the elongated appendages. Arrowhead points to large space between two appendages. Abbreviations: ap, appendage; f, filament; le, ledge; sta, lateral staminode; sti, stigma; sty, style. Scale bar = 100 μm (A–F, I, J), 200 μm (G, H, K–P).

Globba *species with six anther appendages*

Some species (*G. siamensis*, *G. atrosanguinea*) are characterized by the possession of an additional smaller appendage between the two larger appendages seen in species with four appendages. The distal surface of the young anther of this type is not as smooth as that of the other types and is covered with irregular bumps (Fig. 6A, B, I, J). Two narrow elongated ledges develop first on the margins of the connective. Four flattened extensions emerge almost simultaneously in the middle (medio-proximal) part of the anther on these ledges (Fig. 6C, D, K, L). They grow nearly equally, developing into four prominent appendages (Fig. 6E, F, M, N). As in other species, the filament curves in its upper part and the four appendages are pressed against the lower section of the filament. As the four large appendages begin to embrace the filament, two small appendages emerge on the ledges between the four first-formed appendages (Fig. 6G, O). They enlarge less than the other appendages and remain smaller in the mature flowers (Fig. 6G, H, O, P). The smaller appendages arise independently of the other appendages if there is a sufficiently large space on the ledge between the two other appendages (Fig. 6E, F, G). In all samples of *G. siamensis*, there were six appendages and there was a considerable space on the ledge between the larger appendages (Fig. 6E, F). In *G. atrosanguinea*, the presence of six appendages was variable, not all anthers having the extra appendages. The space between the larger appendages was often shorter than in *G. siamensis* (Fig. 6L, N, O).

Anther morphology of Gagnepainia *and* Hemiorchis

The anther morphology of the two *Gagnepainia* spp. is similar in the possession of an apical connective appendage (Fig. 7A–C). In young anthers of *Gagnepainia* (Fig. 7B, C), two narrow ledges develop parallel to the margin of the anther. At later stages of development in *G. godefroyi*, each ledge is flanked towards the connective by a prominent stripe covered with cylindrical trichomes (Fig. 7C, D). This stripe of trichomes is missing from *G. harmandii*, which also differs by its prominent apical extension of the connective (Fig. 7A, B). The anthers of *H. burmanica* are rectangular with two short apical extensions above the thecae (Fig. 7E, F). Abaxial ledges are less prominent, but the stripes of trichomes are more developed (Fig. 7F).

Reconstruction of ancestral character states

We mapped three characters concerning the number and occurrence of appendages (Table 1) on a phylogenetic tree based on the combined ITS/*matK* tree of Williams *et al.* (2004) incorporating results obtained from the MSc thesis of Pospíšilová (2012). In addition to the data reported here, we included appendage numbers from two species (*Globba cernua*, *G. spathulata*) observed by Box & Rudall (2006, figs 8, 9) and from *G. sessiliflora* from Williams *et al.* (2004, fig. 3). *Globba cernua* has four appendages, *G. spathulata* has two and *G. sessiliflora* lacks appendages (Table 1). The Nexus file used in these analyses is available as Supporting Information (S1).

We first analysed the presence versus absence of anther appendages using maximum likelihood (Fig. 8, Supporting Information S5) and parsimony (Supporting Information, Fig. S2). The results of the two analyses were similar. The maximum likelihood analysis showed a high likelihood of anther appendages arising in the immediate ancestor of *Globba* (0.94 presence/0.06 absence) and its subsequent loss in *G. sessiliflora* and *G. racemosa* [subgenus *Mantisia* (Sims)

K.J.Williams section *Haplanthera* (Horan.) Petersen; Fig. 8]. The parsimony reconstruction showed the same pattern, with unequivocal reconstruction of the ancestral character states at both nodes.

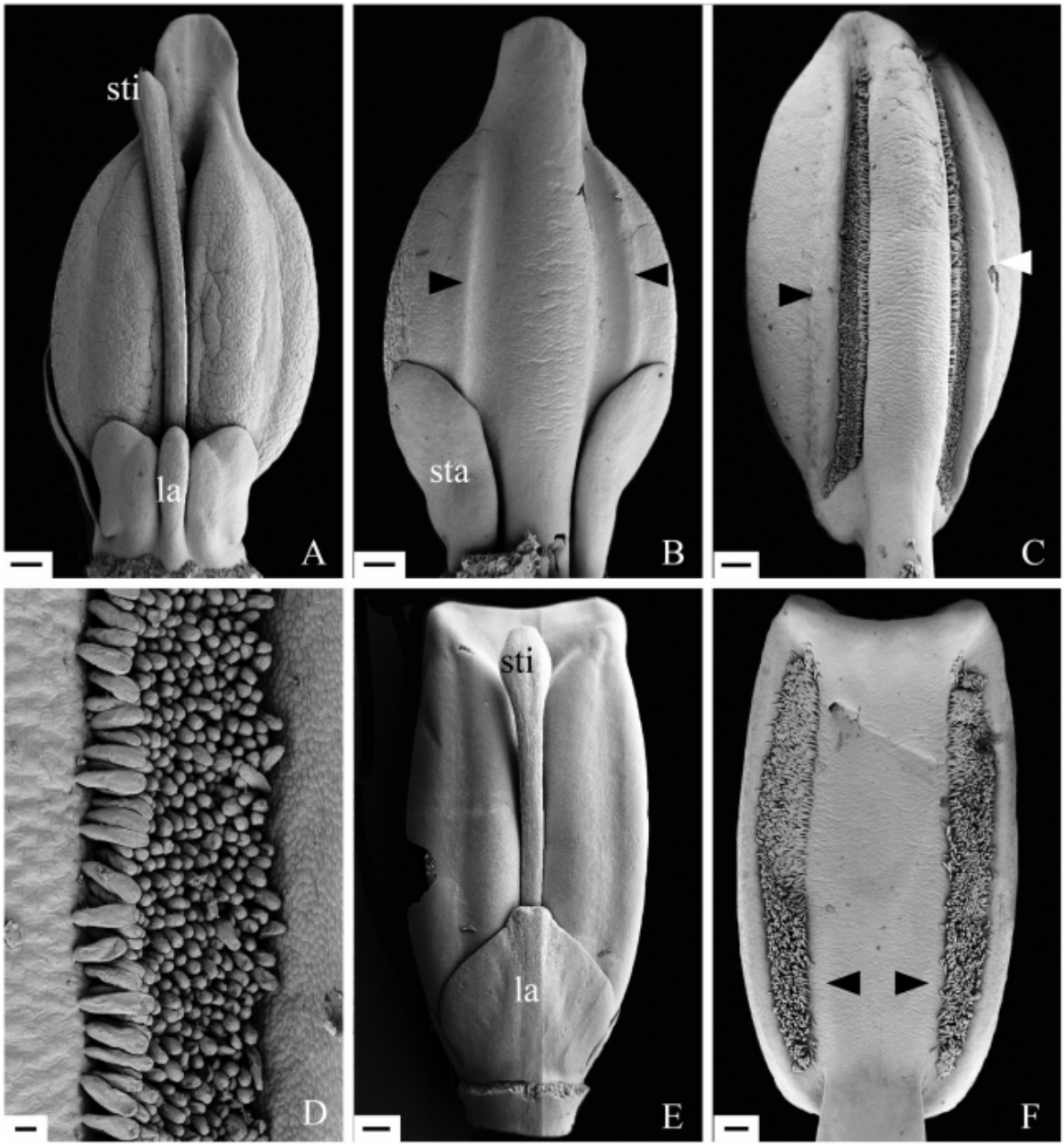

Figure 7. Morphology of anther appendages in *Hemiorchis* and *Gagnepainia*. A–B, *Gagnepainia harmandii*, C–D, *Gagnepainia godefroyi*, E–F, *Hemiorchis burmanica.* A–B, adaxial and abaxial view of young anther before curvature of the filament. Note trilobed labellum and ledges (arrowheads). C, Abaxial view of mature anther showing glandular trichomes and ledges (arrowheads). D, Detail of a stripe with glandular trichomes. E–F, Adaxial and abaxial views of mature anther. Note glandular trichomes and weakly developed ledges. Abbreviations: la, labellum; sta, lateral staminode; sti, stigma; sty, style. Scale bar = 20 μm (D), 100 μm (E–F), 200 μm (A–C).

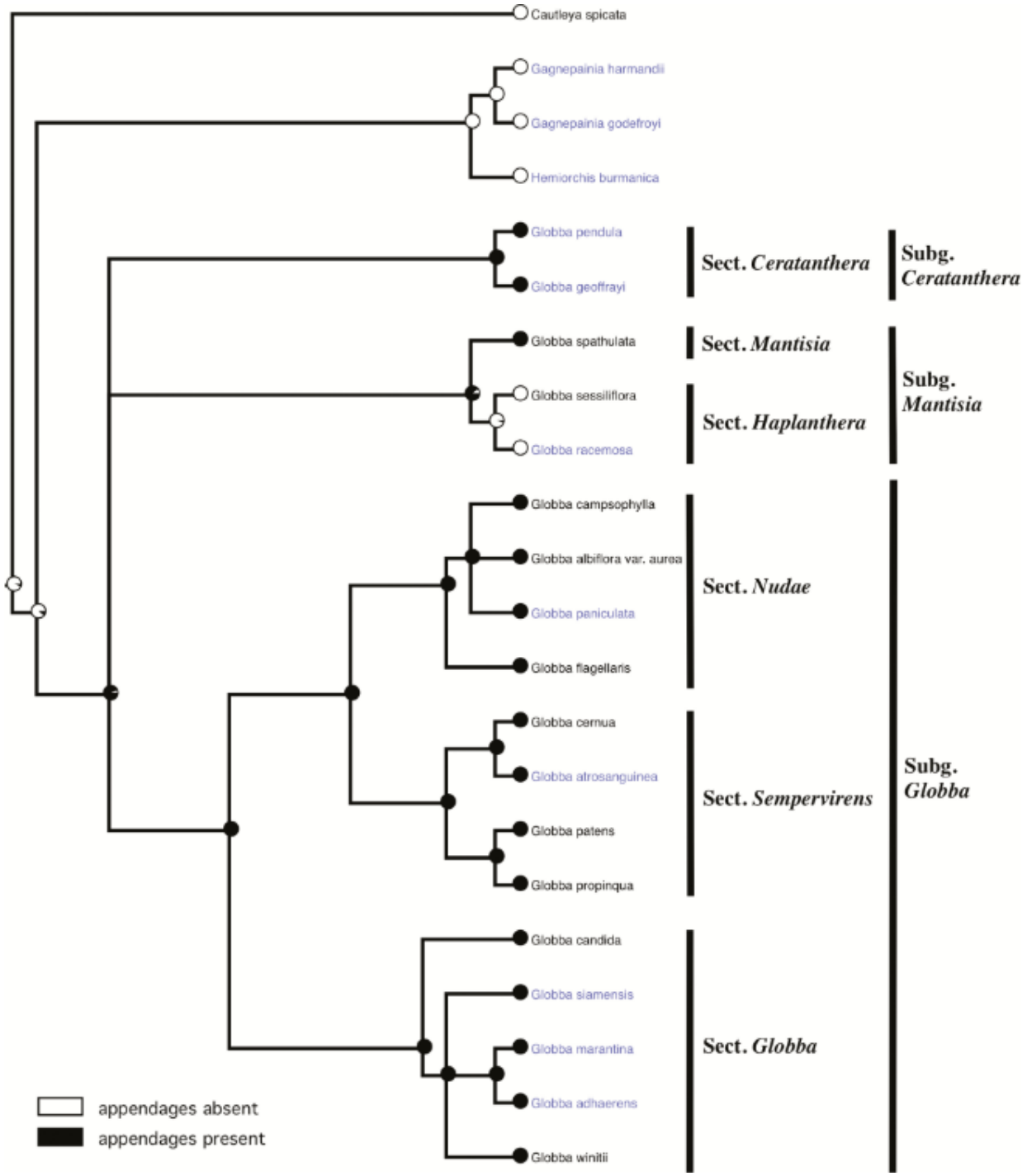

Figure 8. Maximum likelihood reconstruction of presence versus absence of anther appendages in Globbeae with characters unordered. Phylogeny based on the combined ITS/*matK* analysis of Williams *et al.*(2004) supplemented with the ITS phylogenetic data of Pospíšilová (2012) for species not covered by Williams *et al.* Information of *G. spathulata* and *G. cernua* from Box & Rudall (2006). Sections and subgenera according to Williams *et al.* (2004). The likelihood for character states at internal nodes is shown as pie charts (for maximum likelihood support values see Supporting Information S5). Taxa for which the floral development has been investigated are shown in blue.

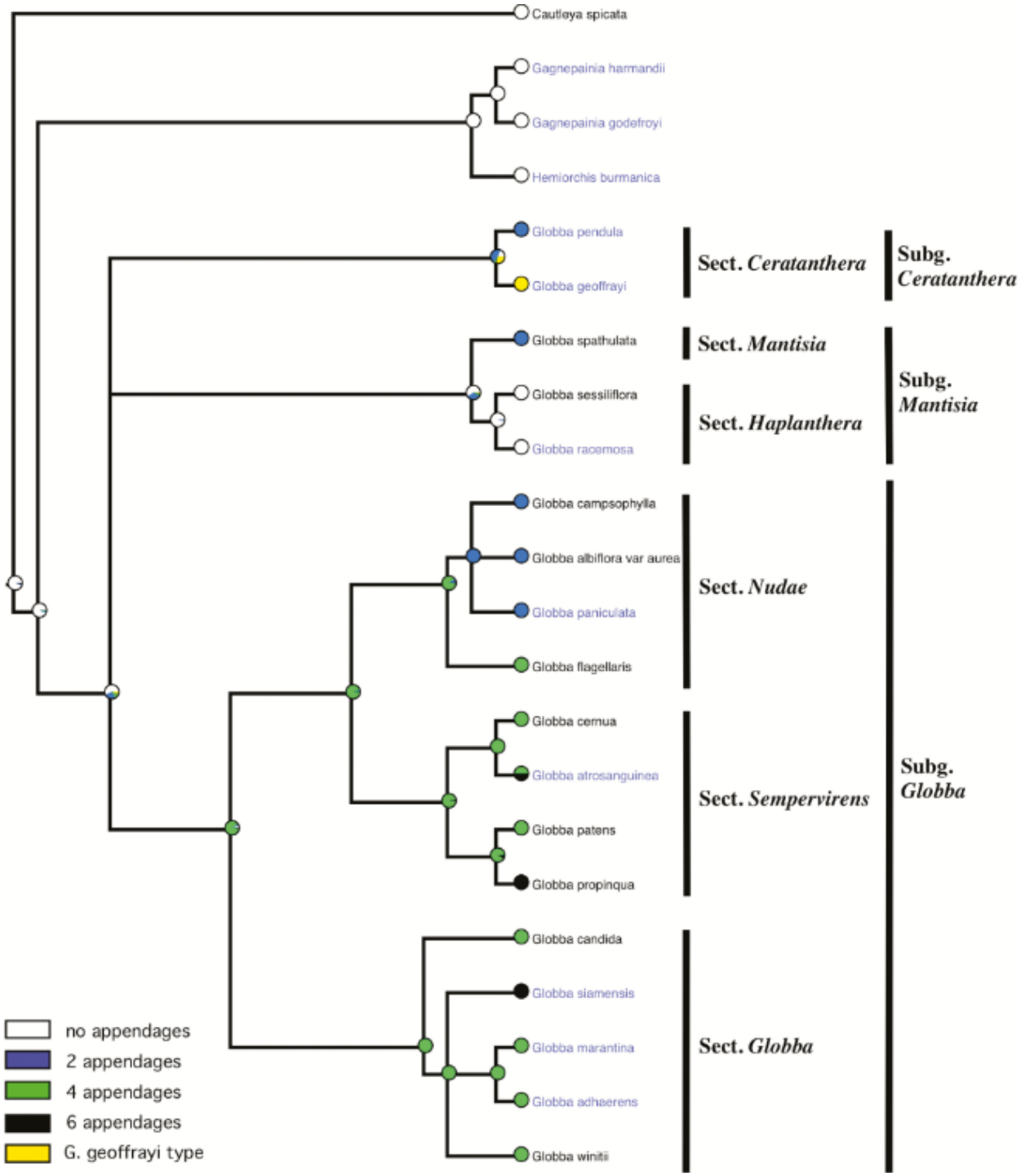

Figure 9. Maximum likelihood reconstruction with unordered character states of anther appendage numbers in the investigated species of *Globba* on a reconstructed phylogeny based on the combined ITS/*matK*analysis of Williams *et al.* (2004) supplemented with the ITS phylogenetic data of Pospíšilová (2012). The character states are: no appendages; two appendages; four appendages; six appendages; *G. geoffrayi* type. Sections and subgenera according to Williams *et al.* (2004). The likelihoods for character states at internal nodes are shown as pie charts (for maximum likelihood support values see Supporting Information S6). Taxa for which the floral development has been investigated are shown in blue.

We also plotted the number of anther appendages using maximum likelihood (Fig. 9, Supporting Information S6) and parsimony with unordered character states (Supporting Information Fig. S3). The two methods again gave similar results but conflict somewhat with the presence/absence reconstructions (Fig. 8). This conflict is addressed more fully below. The maximum likelihood analysis shows that there is a 0.57 likelihood of the ancestor of *Globba* possessing no appendages and a 0.43 likelihood that it possessed one of the other states (Fig. 9, Supporting Information S6). The parsimony analysis shows an equivocal result at this node, with zero and two appendages being possible (Supporting Information, Fig. S3). In both analyses, the ancestor of subgenus *Globba* has four appendages (0.89 likelihood, Fig. 9), with six appendages evolving independently in *G. siamensis*, *G. propinqua* and *G. atrosanguinea*. Both analyses also suggest a reversion from four to two appendages in section *Nudae* subsection *Mediocalcaratae* (*G. campsophylla*, *G. albiflora* var. *aurea*, *G. paniculata*). The ancestor of subgenus *Mantisia* (*G. sessiliflora*, *G. racemosa*, *G. spathulata*) has no appendages by a small margin in the likelihood analysis (0.59 likelihood) and the reconstruction at this node is equivocal in the parsimony analysis. If appendages are lacking at this node, there must have been an independent origin of two appendages in *G. spathulata*. The number of appendages in the ancestor of subgenus *Ceratanthera* (Horan.) K.J.Williams (e.g. *G. pendula*, *G. geoffrayi*) is ambiguous in both analyses.

To resolve the ambiguity in the ancestor of subgenus *Ceratanthera* and address the conflict between the presence/absence and appendage number analyses, we created a new character based on appendage development and plotted this character on a tree consisting only of the species for which we had developmental data. We created both likelihood (Fig. 10, Supporting Information S7) and parsimony (Supporting Information, Fig. S4) plots. The developmental character states in these plots were the same as their mature states (Table 1) with the following exceptions. *Globba geoffrayi* was coded as having two appendages, as its lower two appendages develop significantly earlier than the upper two. The two six-appendaged species (*G. propinqua*, *G. siamensis*) were coded as having four appendages, as the final two of the six appendages develop significantly later than the initial four. The developmental character thus provides a record of the early stages of appendage development. Including information from development resolves the ancestor of subgenus *Ceratanthera* (e.g. *G. pendula*, *G. geoffrayi*) as two-appendaged in both likelihood (0.89 likelihood: Fig. 10, Supporting Information S7) and parsimony analyses (Supporting Information, Fig. S4). The ancestral states on the other parts of the tree are unchanged from the previous analysis, although the likelihoods are slightly different. Recoding *G. geoffrayi* as possessing two appendages on the full tree increases the likelihood that the ancestor of the genus had two appendages (0.50 likelihood) over no appendages (0.39 likelihood) (character plots not shown, but see the Nexus file Supporting Information S1). This coding allows us to treat the condition in *G. geoffrayi* as both autapomorphic for the possession of two large and two small appendages (as in Williams *et al.* 2004) and to show its clear derivation from the two-appendaged condition as demonstrated by our developmental evidence.

The presence of appendages is clearly dependent on the presence of ledges as all species that have appendages also have ledges, but the presence of ledges does not always predict the presence of appendages. *Globba racemosa* and *Hemiorchis burmanica* possess ledges, although they are weakly developed, but lack appendages. More strongly developed ledges are present in *Gagnepainia godefroyi* and *Gagnepainia harmandii*, which also lack appendages (Fig. 7A–C).

The presence of ledges in all species of *Globba* studied and in the outgroup (*Gagnepainia*, *Hemiorchis*) makes it highly likely that the presence of ledges is a primitive character in the genus and that anther ledges will be found in other species of *Globba*, whether or not they possess appendages.

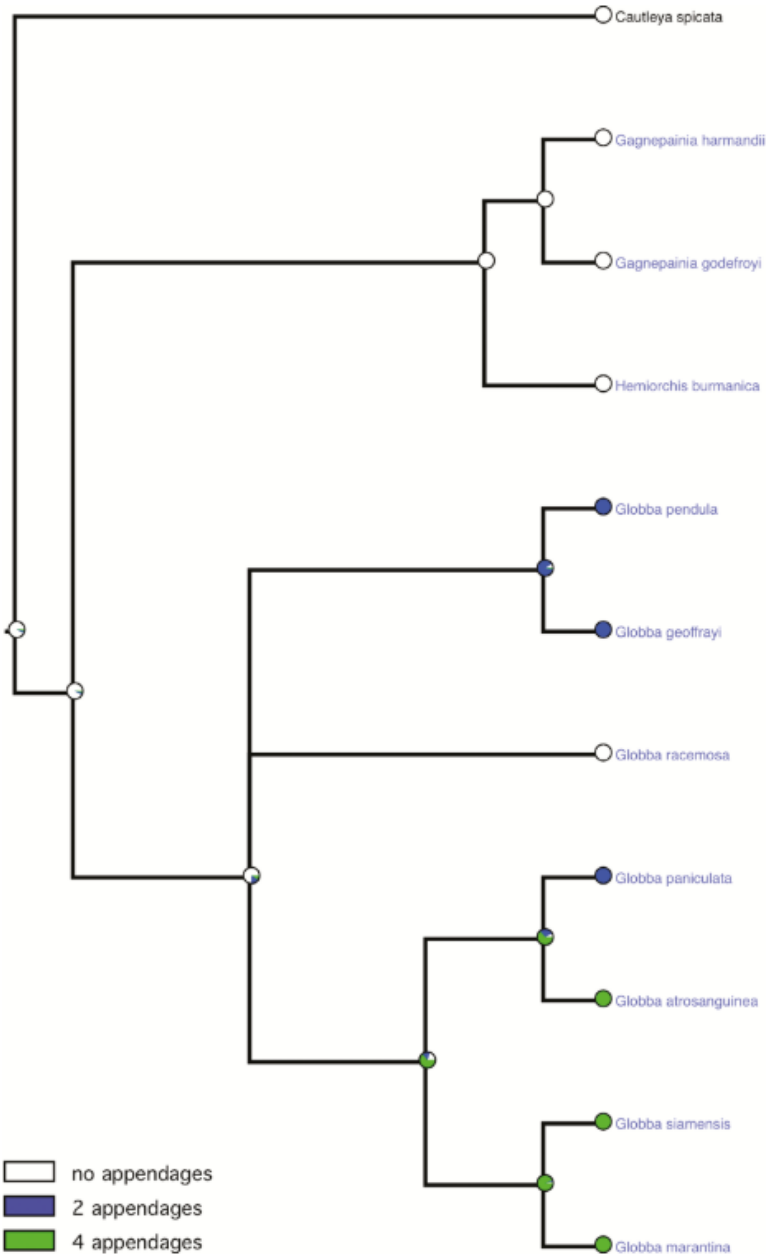

Figure 10. Maximum likelihood reconstruction of the number of appendages at an early stage of development plotted on a phylogenetic tree based on the combined ITS/*matK* analysis of Williams *et al.* (2004)supplemented with the ITS phylogenetic data of Pospíšilová (2012) containing only those species for which we have developmental data. The character states are: no appendages; two appendages (including the *G. geoffrayi* type) and four appendages (including species with six mature appendages). The likelihoods for character states at internal nodes are shown as pie charts (for maximum likelihood support values see Supporting Information S7).

DISCUSSION

Function and development of anther appendages

Anther appendages are conspicuous structures in most *Globba* spp. In bud, the appendages embrace the filament, which becomes elongated and is curved at anthesis. As a result, the style becomes stretched as the string of a bow (Endress, 1994). The function of the appendages is unknown, but Endress (1994) and Williams *et al.* (2004) suggested that they might function as a lever, increasing the likelihood that the anther is placed in a favourable position when approached by a pollinator from the side. Müller (1931) described the mechanism of pollen deposition in *G. atrosanguinea*, which is linked to the strong connection between anther and style. The filament narrows just below the anther and develops a row of collenchymatous cells on one side (Müller, 1931). This point functions as a hinge allowing the movement of the anther in the abaxial direction under pressure of a visiting pollinator and the appendages provide a larger contact surface. In contrast, Fan & Li (2016) found that the tail-like anther crest of *Zingiber densissimum* S.Q.Tong & Y.M.Xia forces the pollinator to adopt a specific position for entry into the flower, thereby enhancing the pollination process. It is plausible that the anther appendages of *Globba* play a similar role in guiding the insect into the right position for pollen transfer. Preliminary observations of flower visitations support this interpretation (Angvirotjanaphat & Newman, personal observation).

Our developmental observations are consistent with the developmental study of Box & Rudall (2006), which showed that appendages arise in the later stages of floral development when the anthers are already well developed. In our study, anther development is similar until the moment the filament starts elongating and curves at the level of the anther. In all species with appendages, the appendages arise on a ledge developing at the margin of the broad connective. Species of *Globba* (*G. racemosa*), *Hemiorchis* and *Gagnepainia* without appendages also possess ledges, but appendages are not formed from these ledges. The outgroup *C. spicata* lacks appendages and may also lack ledges, but there is no clear evidence for this as the scanning electronmicrographs of Ngamriabsakul (2005) do not show the backs of the anthers. Ledges have been informally reported in several other genera of Zingiberaceae that lack appendages (Leong-Škorničková, pers. comm.).

Globba racemosa, which lacks appendages, possesses a broad band of unicellular trichomes parallel to the thecae, between the ledge and the connective (Fig. 3E–L). Similar abundant tufts of trichomes were observed in the outgroup genera *Hemiorchi*s (Fig. 7E, F) and *Gagnepainia* (Fig. 7C, D). Other *Globba* spp. that we investigated have similar trichomes, but they are inconspicuous and are less abundant (Figs 4J, 5J, 6D). It is not known whether there is a correlation between a lower number of trichomes and the development of appendages, or what function these trichomes play in floral ecology. Box & Rudall (2006) mentioned similar glandular trichomes in the species they studied, being most prominent in *G. spathulata*, a species with two appendages relatively close to *G. racemosa*. Box & Rudall (2006) suggested that the glandular trichomes may function in much the same way as the viscidial discs of orchids in attaching pollen to insects, although they stated that this interpretation was speculative. Similarly, Moyano, Cocucci & Sérsic (2003) suggested that the glandular trichomes on the anther of *Leonurus sibiricus* L. (Lamiaceae) play a comparable role in pollination.

Although appendage development is associated with the presence of a prominent ledge, there is no correlation between the presence of a ledge and the number of appendages formed. In two-appendaged species, the appendages develop from the lower part of the ledge and expand distally over the length of the anther (Fig. 4B, C, H, I). With four appendages, two medium-sized appendages develop in the proximal half of each ledge and enlarge with the anther to cover the full length of the ledges (Fig. 6I–K, N, O). As a result, the relative size of each appendage is smaller than in anthers with two appendages. Two- and four-appendaged anthers thus arise from developmental patterns that differ from inception. Two-appendaged anthers are not four-appendaged species that have lost their distal or proximal two appendages, as suggested by Williams *et al.* (2004).

In species with six appendages, there is a greater gap between the two first-formed appendages, allowing room for the development of the final two appendages. The distal appendages and the proximal appendages are quite broad and wide, and the two middle appendages are much smaller and might be easily overlooked. For instance, Müller (1931) showed the six appendages in her drawings of *G. atrosanguinea* without mentioning them in the text. The presence of six appendages was found to be consistent in one of our study species (*G. siamensis*, Fig. 6G, H) and variable in another (*G. atrosanguinea*, Fig. 6N, P).

Four- and six-appendaged stamens occasionally have secondary appendages (lobes) borne on the larger primary appendages (Fig. 2O, P, 5L, 6H), something that is never seen in the two-appendaged species. The late development of these lobes shows that it is possible to separate the occasional dentations of appendages from the more consistent occurrence of six appendages. The occurrence of six appendages is relatively stable among six-appendaged species (Fig. 6A–C, I, J), whereas dentations can be variable. We therefore describe six appendages as a type separate from the four-appendaged anthers.

Appendage structure and development in *G. geoffrayi* are different from all other species with four appendages. In mature anthers, the two distal appendages are much smaller than the proximal ones, making the appendages of *G. geoffrayi* unique in our study (Fig. 5F). In other four-appendaged species, the four appendages emerge almost simultaneously on the ledges, but in *G. geoffrayi* the distal appendages emerge from the ledge later than the proximal ones. Their development is delayed compared to four-appendaged species. However, the early developmental stages of *G. geoffrayi* are remarkably similar to those of two-appendaged species. The distal appendages thus appear as a developmental addition to the two-appendage type and have been treated as such in our character plots. By addition, we mean a further continuation of the developmental process beyond what is seen in the two-appendaged species (Hufford, 2001; Ronse De Craene, 2018).

Systematic implications of the evolution of anther appendages

Anther appendages play an important role in the classification of *Globba*. Since the first infrageneric classification by Horaninow (1862), circumscription in *Globba* has focused primarily on the number of appendages on the anther. Schumann (1904)recognized three sections according to the number of anther appendages: section *Haplanthera* (no appendages),

section *Ceratanthera* (Horan.) Petersen (two appendages) and section *Marantella* (Horan.) Benth. & Hook.f. (four appendages; now called section *Globba*). Using the insertion point of the two appendages, Schumann (1904) subdivided section *Ceratanthera* into three series, *Basicalcaratae* Schum., *Mediocalcaratae* Schum. and *Apicicalcaratae* Schum. Larsen (1972) created the monospecific section *Nudae* Larsen for a species of northern Thailand (*G. nuda*K.Larsen) with highly reduced bracts and leaves, but which would otherwise correspond to section *Globba* (i.e. with four anther appendages). Williams *et al.*(2004) proposed a revised classification of *Globba sensu lato* (including *Mantisia*) with three subgenera, seven sections and two subsections (partly shown in our Figs 8 and 9).

Williams *et al.* (2004) discussed appendage homology and evolution at some length and hypothesized three possible evolutionary patterns. These suggestions were based on the hypothesized homology between the upper two appendages of four-appendaged species with the two appendages of section *Nudae* subsection *Mediocalcaratae* (e.g. *G. paniculata*, Fig. 2C), and of the lower two appendages with the two appendages of subgenus *Ceratanthera* (e.g. *G. pendula* and *G. geoffrayi*, Fig. 2F, H). These homologies are not supported by our developmental study. The hypothesized evolutionary patterns are: (1) two basal appendages (e.g. *G. spathulata*) are primitive in the genus, with a secondary loss of appendages in subgenus *Mantisia* (e.g. *G. racemosa*, Fig. 2A, B); (2) two major independent derivations of anther appendages, one in subgenus *Ceratanthera* (e.g. *G. geoffrayi*) and one in subgenus *Globba* (e.g. *G. marantina*, *G. siamensis*, *G. atrosanquinea*, Fig. 2O, Q, T), with losses and modifications of appendages occurring in other parts of the genus and (3) four independent derivations of appendages in the genus, with one or two losses in different sections of subgenus *Globba* (e.g. *G. paniculata*, Fig. 2C). Only the first of these hypotheses finds any support in our study, although we are able to provide only weak support for two appendages being primitive in the genus.

Mapping the presence/absence of anther appendages on a tree based on Williams *et al.* (2004) and Pospíšilová (2012) shows a high likelihood that appendages are a plesiomorphic feature of the genus (Fig. 8). Coding the character states based on the number of appendages, with *G. geoffrayi* coded as possessing a unique state, produces an equivocal result for subgenus *Ceratanthera* (e.g. *G. pendula* and *G. geoffrayi*) and fails to resolve a clear ancestral state for the genus (Fig. 9). Incorporating developmental evidence allows us to resolve the ancestor of subgenus *Ceratanthera* as possessing two appendages (Fig. 10) and results in a character map that reconstructs the ancestor of the genus with two appendages by a slight margin (0.50 likelihood over 0.39 likelihood for no appendages).

All of our reconstructions show a loss of appendages in subgenus *Mantisia* section *Haplanthera* (e.g. *G. racemosa*) and a primitive state of four appendages in subgenus *Globba*. Six appendages appear to have evolved independently in subgenus *Globba*section *Globba* (e.g. *G. atrosanguinea*, *G. propinqua*) and subgenus *Globba* section *Sempervirens* (e.g. *G. siamensis*) (Fig. 9). Our plots also show a reversion from four to two appendages in subgenus *Globba* section *Nudae* subsection *Mediocalcaratae* (e.g. *G. paniculata*). The anther morphology of *G. geoffrayi* appears unique, but is clearly derived from a two-appendaged ancestor as shown by developmental evidence and supported by character plots.

The lack of support for a resolved topology of the three subgenera (*Ceratanthera*, *Mantisia* and *Globba*) at the base of the genus (Figs 8 and 9; Williams *et al.* 2004, fig. 11) could be taken to make an unequivocal interpretation of appendage evolution difficult. However, placing subgenus *Mantisia* as the sister group to subgenus *Globba*, as suggested by Williams *et al.* (2004), but not supported in their strict consensus tree, does not significantly change the likelihoods reported here.

Our data demonstrate that two- and four-appendaged species can be distinguished developmentally, but developmental evidence, taken on its own, is equivocal on the question of the evolution of these character states. Our character plots provide weak evidence that two-appendaged anthers are the evolutionary precursors of four-appendaged anthers, but it is possible, although slightly less likely, that four-appendaged species evolved directly from species with no appendages. If there was an increase from two to four appendages, it may be linked with the bifurcation of the single outgrowth in the two-appendaged species to produce the double outgrowths of four appendages (compare Fig. 4B and 5H). However, it could also be that both two- and four-appendaged anthers arose directly from species with no appendages. Development alone does not allow us to decide between these alternatives. Developmental evidence is, however, unequivocal in its support for the derivation of six appendages from four.

CONCLUSIONS

We confirm the strong link between the morphology of anther appendages and the sections defined by Williams *et al.* (2004). Anther morphology appears to be highly similar among *Globba* spp., notwithstanding the fact that there are six, four, two or no appendages present in different species. Our analysis shows that appendages were present in the ancestor of *Globba*, with weak support for two appendages being the ancestral state. Four appendages evolved in the ancestor of subgenus *Globba*; two most likely occurred in the ancestor of subgenus *Ceratanthera*; and the ancestor of subgenus *Mantisia* most likely had no appendages. Additionally, the sister genera *Hemiorchis* and *Gagnepainia* are shown to lack appendages but there is clear evidence of a ledge in these genera, with a well-developed ledge in *Gagnepainia*. It is possible that the possession of ledges is an apomorphy of the tribe Globbeae (*Globba*, *Gagnepainia*, *Hemiorchis*) although ledges have been reported elsewhere in the family.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

S1-Globba.nex. Nexus file used in the analyses. The file contains two trees. The topology of both trees is based on the combined ITS/*matK* analysis of Williams *et al.*(2004) supplemented with the ITS phylogenetic data of Pospíšilová (2012). Tree 1 is used in Figs 8, 9, S2, S3, S5 and S6. Tree 2 is used in Figs 10, S4 and S7. Character 1 is presence/absence of appendages. Character 2 is number of appendages, with *Globba geoffrayi* coded as having a unique state (*G. geoffrayi* type). Characters 1 and 2 are for use with Tree 1. Character 3 uses the same coding as Character 2 except that the two six-appendaged species are coded as having four appendages and *G. geoffrayi* is coded as having two. This coding represents the character states during early development. Character 3 can be used with either Tree 1 or 2.

Figure S2. Parsimony reconstruction of presence/absence of anther appendages in the investigated *Globba* spp. on a phylogenetic tree based on the combined ITS/*matK*analysis of Williams *et al.* (2004) supplemented with the ITS phylogenetic data of Pospíšilová (2012).

Figure S3. Parsimony reconstruction with unordered character states of anther appendage numbers in the investigated *Globba* spp. on a reconstructed phylogeny based on the combined ITS/*matK* analysis of Williams *et al.* (2004) supplemented with the ITS phylogenetic data of Pospíšilová (2012). The character states are: no appendages; two appendages; four appendages; six appendages; *G. geoffrayi* type.

Figure S4. Parsimony reconstruction with unordered character states of the number of appendages at an early stage of development on a reconstructed phylogeny based on the combined ITS/*matK* analysis of Williams *et al.* (2004) supplemented with the ITS phylogenetic data of Pospíšilová (2012) containing only those species for which we have developmental data. The character states are: no appendages; two appendages (including the *G. geoffrayi* type); four appendages (including species with six mature appendages).

Figure S5. Information linked to maximum likelihood of character evolution along nodes shown in Fig. 8.

Figure S6. Information linked to maximum likelihood of character evolution along nodes shown in Fig. 9.

Figure S7. Information linked to maximum likelihood of character evolution along nodes shown in Fig. 10.